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Differential effect of $CD4^{+}Foxp3^{+}$ T-regulatory cells on the B and T helper cell responses to influenza virus vaccination

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ABSTRACT

The T-regulatory (T-reg) cells restrict the T-cell functions in various viral infections including influenza infection. However little is known about the effect of T-regs in influenza vaccination. Herein, we found that immunization of BALB/c mice with a prototype of UV-inactivated influenza PR8/A/34 virus vaccine expanded the $CD4^{+}Foxp3^{+}$ T-reg pool and fostered the development of virus-specific $CD4^{+}Foxp3^{+}$ T-reg cells. Increasing the size of $Foxp3^{+}$ T-reg pool did not alter the primary PR8-specific B-cell response, but it did suppress the primary and memory PR8-specific T helper responses induced by vaccination. In contrast, the vaccination-induced T helper cell response was augmented in the absence of $CD4^{+}Foxp3^{+}$ T-reg cells. Since CD4 T helper cells contribute to anti-influenza protection, therapeutic "quenching" of T-reg function prior to vaccination may enhance the efficacy of influenza vaccination.

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1. Introduction

Pandemic outbreaks of the influenza A viruses may result in numerous fatalities around the globe. Effective vaccines represent the best approach to prevent the pandemic spread, but at present the vaccine preparations using inactivated viruses provide limited protective immunity for the upper respiratory tract where the infectious process is first taking place. Therefore, deciphering the mechanisms that restrict anti-influenza protection by vaccination is a prerequisite to improving the efficacy of vaccination.

Influenza viruses are enveloped orthomyxoviruses with a segmented RNA genome of negative polarity, containing eight segments that encode ten proteins in the case of type A and B viruses, and seven segments encoding nine proteins in the case of type C virus [1]. The influenza viruses are divided into three types based on structural differences among the internal proteins, and in subtypes based on differences in the amino acid sequences and antigenicity of their hemagglutinin and neuraminidase proteins. Regardless the influenza virus strain, the hemagglutinin (HA) envelope protein plays an important role in viral entry in the cells and, therefore, is a critical factor of virulence. The anti-influenza viral immunity is a complex process involving both the B- and T-cell compartments of the innate and adaptive immunity. Both the CD4 and CD8 mediated anti-influenza viral responses specific for peptides derived from viral proteins are well presented by MHC class II and class I encoded gene products [2,3] expressed on antigen presenting cells [4].

Mice and individuals infected by influenza virus have a sharp decrease in the number and function of lymphocytes concomitant with an increased number of T-regulatory (T-reg) cells shortly after exposure to the virus [5,6]. A number of reports argue for a suppressogenic effect of T-regs on the anti-viral immune responses [7–10]. Early studies on the mechanisms by which A/Puerto Rico/8/34 [H1N1] influenza virus limit the proliferation of virus-specific cellular immune responses suggested a role of virus-specific CD8a T-cells [11]. Recently, the suppressive mechanism of influenza-specific CD8a T-cells was attributed to IL-10 secretion at the site of inflammation [12]. There is also evidence that $CD4^{+}$ T-regs can limit the delay type of hypersensitivity to intradermal influenza virus inoculation [13]. A significant increase in the number of peripheral $CD4^{+}$ T-reg pool in individuals infected by influenza A (H1N1) virus, particularly in those with pneumonia complications, was recently reported [6].

Naturally born $CD4^{+}(25^{high})$ $Foxp3^{+}$ T-regs maintain the T- and B-cell homeostasis and can also restrict T-cell responses to *self* and foreign antigens [14]. T-regs are critical not only for protection

Abbreviations: HA, hemagglutinin; HAI, hemagglutination inhibition assays; HK, Hong Kong influenza virus strain; MFI, mean fluorescence intensity; PR8, (A/PR/8/34 influenza virus strain); RAG2 KO, RIP-PR8/HArecombination activating gene 2 knockout mice transgenic for the hemagglutinin protein of A/PR/8/34 virus expressed in the pancreatic beta cells under the rat insulin promoter; TCR, T-cell receptor; TCR-PR8/HA, T-cell receptor specific for the $HA_{110-120}$ CD4 T-cell immunodominant epitope of hemagglutinin of A/PR/8/34 influenza virus; T-reg, $CD4^{+}Foxp3^{+}$T-regulatory cells; Tg, transgenic.

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against autoimmunity [15] but also for protection of fetus against maternal immune responses [16], restriction of host allogeneic T-cell responses against graft transplantation [17], suppression of CTL anti-tumor activity in cancer [18], and modulation of Th2 responses in allergic diseases [19]. Recently, T-regs were shown to restrict immune responses against viral, bacterial, and parasitic infections [20–24]. Thus, in chronic Hepatitis C (HCV) viral infection the number of $CD4^+25^+$ $Foxp3^+$ T-regs is increased, whilst T-reg depletion associates with enhanced HCV-specific CD4- and CD8 T-cell responses [25–27]. In Herpex simplex viral infection, depletion of T-regs was followed by a greater migration of T inflammatory cells from the draining lymphoid nodules to the site of infection [28,29]. Data from HIV-infected patients demonstrated that $CD4^+25^+$ T-regs suppress the virus-specific CD4- and CD8 T-cells [30–32], and that HIV-induced T-regs can restrict the infectivity of Cytomegalovirus virus [30]. In contrast to other viral infections, the human T-cell lymphotropic virus type-1 preferentially infects the $CD4^+Foxp3^+$ T-regs and down-regulates their Foxp3 expression, thereby contributing to multi-organ lymphocyte infiltration [33,34]. However, in most cases, T-reg depletion leads to a better control of viral infections in various animal models (reviewed in [35]).

Recent data suggested a restrictive effect of T-reg cells on the delay type of hypersensitivity to influenza infection [10,13], but little is known about their effect on the anti-influenza CD4 T helper cell responses induced by vaccination. Herein, we investigated the effects of $CD4^+Foxp3^+$ T-reg cells on B- and T-cell responses induced by influenza virus vaccination.

2. Materials and methods

2.1. Animals

Naïve BALB/c and BALB/c, RAG2 KO mice (4–5-week-old) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). TCR-PR8/HA transgenic (Tg) mice expressing the 14.3d T-cell receptor that recognizes the $HA_{110-120}$-CD4 T-cell immunodominant epitope of hemagglutinin protein (HA) of the A/PR/8/34 influenza virus [36] were used as source of antigen-specific T-regs and conventional HA-specific CD4 T-cells. RAG2 KO, RIP-PR8/HA Tg mice on a BALB/c background expressing the influenza HA viral protein in pancreatic β-cell islets under the rat insulin promoter [36] were used as cell recipients in adoptive transfer experiments. The TCR-PR8/HA Tg and RAG2 KO, RIP-PR8/HA Tg mice were maintained in our pathogen-free facility at USUHS according to federal and local regulations.

2.2. Immunization protocols

Naïve BALB/c mice enriched or not with syngeneic naïve T-reg cells (sorted $CD4^+CD25^+Foxp3^+$ cells from BALB/c mice), and BALB/c, RAG2 KO mice reconstituted with either total spleen cells or T-reg depleted spleen cells from naïve BALB/c mice were immunized intraperitoneally (i.p.) with a single dose of 200 μg of UV-inactivated type A/PR/8/34 (PR8) or type B HK (BHK) influenza viruses with respect to the protein content as measured by Bio-Rad assay (Bio Rad Laboratories, Hercules, CA, USA). Both influenza viral strains were purified by centrifugation on sucrose gradient (Charles River, North Franklin, CT, USA). T-reg enrichment of BALB/c mice was carried out 3 days before vaccination by intraperitoneal (i.p.) infusion of naïve BALB/c T-regs (6.5×10^6 $CD4^+CD25^+$ cells) sorted on magnetic immunobeads according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA and R&D Systems, Minneapolis, MN, USA). Adoptive cell transfers in BALB/c, RAG2 KO mice with 74×10^6 total spleen cells/mouse or CD25-depleted spleen cells/mouse were carried out 1 day before the i.p. vaccination with 100 μg viral proteins/mouse. On day 8 post-vaccination, spleens were collected and cells were analyzed.

2.3. Cell isolation

Single-cell suspensions of $CD4^+CD25^+$ T-reg cells were negatively sorted to 90–95% purity from the spleen of naïve BALB/c mice on CD4 columns followed by incubation with CD25 immunobeads and enrichment with PE-labeled anti-CD25 Abs coupled to magnetic beads according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA and R&D Systems, Minneapolis, MN, USA). Sorted $CD4^+CD25^+$ T-regs showed Foxp3 expression as determined by FACS intra-nuclear staining with anti-Foxp3 Ab (eBioscience, San Diego, CA, USA).

2.4. Cell proliferation assay

Single-cell suspensions of splenocytes (10^7 cells) from individual mice of each group of mice were incubated with $HA_{110-120}$ synthetic peptide (40 μg/mL) or Con A (2 μg/mL) in flat bottom 12-well plates (Corning, Lowell, MA, USA) for 5 days at 37 °C. Aliquot cultures were transferred in flat bottom 96-well plates for testing the proliferative index using Cell Titer 96® Non-Radioactive Cell Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI, USA). The index of proliferation was determined based on the OD units ($\lambda = 490$ nm) measured in a 96-well plate reader (Molecular Devices Vmax, Sunnyvale, CA, USA).

2.5. Cytokines assay

Single-cell suspensions of splenocytes from individual mice in each group of mice were incubated at 10^6 cells/well with either $HA_{110-120}$ synthetic peptide (20 μg/10^6 cells), Con A (1 μg/10^6 cells), or UV-inactivated PR8 virus preparation (10 μg protein/10^6 cells). Cells were incubated in round bottom 96-well plates at 5% CO_2 and 37 °C for 2 days for IL-2 measurements, and 3 days for IL-4, IL-10, and IFN-γ measurements. Cytokine secretion in cell culture supernatants was assessed by Multiplex mouse cytokines kits using a Luminex instrument according to the manufacturer's instructions (Luminex Corporation, Austin, TX, USA). Cytokine concentrations were calculated based on the acquired mean fluorescence intensity (MFI) using a 5 parameter logistics model equation (MasterplexQT software, Miraibio, San Francisco, CA, USA).

2.6. Immunohistology of pancreas

Pancreata of mice were fixed overnight in 10% phosphate-buffered formalin and embedded in paraffin. Serial paraffin-embedded sections were stained with hematoxylin-eosin, or immunostained with 1:200 dilution of a rabbit anti-insulin antibody (Santa Cruz Biotech, Santa Cruz, CA, USA) and revealed by a 1:5000 dilution of goat anti-rabbit IgG-HRP conjugate (Southern Biotechnologies, Birmingham, AL, USA).

2.7. Adoptive cell transfer experiments

Negatively sorted $CD4^+$ splenic cells from naïve BALB/c mice enriched or not in T-regs and then immunized with PR8 or BHK virus were isolated on CD4 columns (R&D Systems, Minneapolis, MN, USA) 42 days post-immunization, and infused alone, or co-infused i.p. with diabetogenic TCR-PR8/HA splenic cells into RAG2 KO, RIP-PR8/HA Tg mice. The blood glucose level in recipient mice was determined bi-weekly in the blood withdrawn from the tail vein using Accu-Check glucose strips (Roche, Indianapolis, IN, USA). Mice were considered diabetic after two consecutive readings of glycemia higher than 200 mg/dL. In some experiments, BALB/c, RAG2 KO mice were infused i.p. with total spleen cells or T-reg (CD25)-depleted spleen cells from naïve BALB/c mice, and 1 day later were immunized i.p. with 100 μg of UV-inactivated PR8 virus in saline. Eight days post-immunization, the spleen cells were analyzed for cytokine secretion and Foxp3 mRNA expression.

2.8. Antibody assays

Two methods were used to measure the anti-influenza Ab response in immunized mice. First, the ELISA method was carried out on flat bottom 96-well plates coated with PR8 (5 μg/mL) in 0.1 M bicarbonate buffer (pH 9.6) overnight at 4 °C and blocked overnight at 4 °C with 5% BSA in PBS. Mice sera (1/100 dilution in 1% BSA/PBS) were added to the plates for 2 h at 37 °C, washed, and then incubated for 2 h at room temperature either with anti-mouse IgG (H + L) antibody-Biotin conjugate (Abcam, Cambridge, MA, USA), or with anti-mouse IgG1 (Fcγ1) antibody-Biotin conjugate (Jackson ImmunoResearch, West Grove, PA, USA), or with anti-mouse IgG2a (Fcγa) antibody-Biotin conjugate (BD Pharmingen, San Diego, CA, USA). Plates were washed, and bound anti-IgG Ab-Biotin conjugates were revealed by a streptavidin-HRP conjugate (Jackson ImmunoResearch) developed in 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BD Biosciences, San Jose, CA, USA). The OD units corresponding to the antibody titers were measured at $\lambda = 450$ nm in flat bottom 96-well plates in an ELISA reader instrument (Molecular Devices Vmax, Sunnyvale, CA, USA), and then expressed as μg/mL based on a calibration curve built with a PR8-specific IgG1 Ab (#PY102) as we previously described [37]. Secondly, the PR8 and BHK neutralizing Ab titers were measured by Hemagglutination Inhibition Assay (HAI) in 96-well plates as we previously described [37]. Briefly, sera from individual mice of each group of mice (25 μl) were pre-incubated overnight at 37 °C in 1 mL of phosphate buffer (pH 7.4) containing 5 mM CaCl2, and neuraminidases from Arthrobacter ureafaciens and Colstridium perfrigens (50 mU each) (Calbiochem, Gibbstown, NJ, USA). Serial serum dilutions were further incubated for 2 h at room temperature with 1% sheep red blood cells (50 μl/sample) in saline (Innovative Research, Novi, MI, USA) in the absence of presence of 25 μl of sucrose gradient-purified PR8 or BHK virus (20 μg viral protein/mL). The HAI titers were expressed as the one above the first serum dilution showing inhibition of hemagglutination.

2.9. Flow cytofluorimetry

Single-cell suspensions of splenocytes (10^6 cells) from different groups of mice were stained for 30 min at 4 °C using various antibody-dye conjugates and their corresponding isotype controls, according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). The mean fluorescence intensity (MFI) was measured by FACS at the single-cell level in 100,000 cell events acquired by a LSR II Becton-Dickinson instrument coupled to the WINLIST software (Verity, Topsham, ME, USA).

2.10. Real-time RT-PCR

Total RNA and cDNA from CD4$^+$ splenocytes were prepared using NucleoSpin RNA II kit (BD Biosciences Clontech, Palo Alto, CA, USA) and, respectively, Qiagen One Step RT-PCR kit (Qiagen Inc., Valencia, CA, USA). Some 500 ng RNA was used to synthesize the first cDNA strand following the manufacturer's protocol. The primers for murine Foxp3 were as follows: (forward) 5′CAGCTGCCTACAGTGCCCCTAG3′ and (reverse) 5′CATTTGCCAGCAGTGGGTAG3′ [38]. Specific primers for T-bet, STAT4, STAT6, cMAF, and GATA-3 were purchased from Applied Biosystems. Measurements of gene products were carried out as previously described [39]. Quantitative RT-PCR measurements were performed on an ABI Prism 7700 with SDS 1.9.1 software (Applied Biosystems), and the relative mRNA levels were estimated using 18s rRNA as reference (Applied Biosystems, Foster City, CA, USA).

2.11. Biostatistics

Significance of individual differences in the mean fluorescence intensity (MFI, cell density) of Foxp3 and CD44 protein expression in T-reg and memory CD4$^+$ T-cells from different groups of mice, as well as individual variations in the virus-specific Ab and HAI titers, was determined by the non-parametric Student's *t*-test. p^* values lower than 0.05 were considered significant. The intra-assay differences in cytokine secretion and the proliferation index of T-cells in triplicate cultures were expressed as mean ±standard deviation (SD) at 99% interval of confidence. The relevance of differences in survival and diabetes incidence of RAG2 KO, RIP-PR8/HA mice infused with T-cells from virus-immunized BALB/c mice, or co-infused with diabetogenic TCR-PR8/HA Tg T-cells, or infused only with diabetogenic TCR-PR8/HA Tg T-cells alone (diabetes control group) was estimated by Kaplan–Meier test.

3. Results

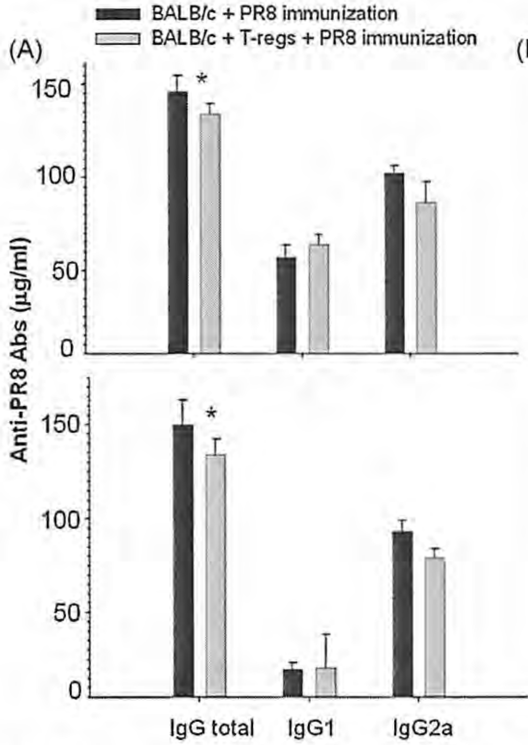
3.1. Foxp3$^+$ T-reg cells do not alter the B-cell responses to influenza vaccination

Reports indicated that T-reg cells exert inhibitory effects on the anti-viral immune responses. Herein, we investigated whether the size of CD4$^+$Foxp3+ T-reg pool may influence the humoral response to PR8 influenza virus by comparing the anti-PR8 viral antibody titers in naïve vs. T-reg-enriched, BALB/c mice immunized with a UV-inactivated A/PR/8/34 vaccine prototype. Although T-reg enrichment in BALB/c mice (6.5×10^6 splenic T-reg cells from naïve BALB/c mice) 3 days prior to immunization increased the size of CD4$^+$Foxp3$^+$ T-reg pool *in vivo* from 2.8 to 4.5%, both groups of mice showed similar kinetics of virus-specific antibody titers 14 and 42 days post-immunization (Fig. 1). Thus, the titers of total PR8-specific IgG, IgG1, and IgG2a antibodies were not significantly altered 14 and 42 days post-vaccination in animals supplemented with T-reg cells; $p = 0.25$ for IgG and IgG2a, and $p = 0.15$ for IgG1 Abs between groups of mice enriched or not in T-reg pool prior to PR8 vaccination.

The anti-influenza neutralizing antibodies specific for PR8 hemagglutinin (HA) protein are critical for virus clearance from the lungs. Thus, measuring the hemagglutination inhibition Ab titers (HAI) is a relevant test for measuring protective (neutralizing) antibodies produced during the primary B-cell response induced post-vaccination or during influenza viral infection. The HAI Ab titers measured 14 days after PR8 immunization were in a 1/320 to 1/640 range, as compared with mice supplemented with T-regs prior to immunization (1/240–1/160) (Fig. 1B). Forty-two days post-immunization, the HAI Ab titers were comparable, but slowly declined in both groups of mice (1/160–1/320 vs. 1/60–1/240). These results indicated that variations in the size of Foxp3$^+$ T-reg pool do not significantly affect the primary PR8-specific B-cell response induced by vaccination.

3.2. Foxp3$^+$ T-reg cells suppress the primary and memory T helper cell responses to influenza vaccination

Although anti-influenza neutralizing antibodies can protect against influenza infection in the absence of T-cells, the influenza-specific CD4 T-cells were shown to partially clear the virus from the lungs independently of antibodies. At the same time, influenza-specific CD4 T memory cells play a major role in recalling the



(B)

Group of mice:	PR8 virus HI titers:
PR8-immunized and analyzed 14 days post-immunization	1/320-1/640
T-regs infused prior to PR8-immunization and analyzed14 days post-immunization	1/240-1/640
PR8-immunized and analyzed 42 days post-immunization	1/160-1/320
T-regs infused prior to PR8-immunization and analyzed 42 days post-immunization	1/160-240

Fig. 1. Effect of T-reg enrichment on B-cell responses upon PR8 viral vaccination. (A) Groups of BALB/c mice ($n = 6$) were infused or not 3 days prior to i.p. PR8 immunization (200 µg viral protein/mouse) with $CD4^+CD25^+$ T-reg cells (6.5×10^6 cells per mouse). Fourteen days (top) and forty-two days later (bottom), the anti-PR8 total serum IgG, IgG1, and IgG2a levels were measured by ELISA at 1/100 dilution using PR8 virus-coated plates (5 µg protein per mL) as described. Results are expressed as geometric mean of µg/mL ±SD between sera from individual mice calculated based on the OD values ($\lambda = 450$ nm) obtained in ELISA, and a calibration curve using an anti-PR8 IgG Ab. (B) Hemagglutination Inhibition Assay (HAI) was performed for the same groups of mice like in panel A. The HAI Ab titer was assigned as the last inhibitory dilution of serum. Shown is one of two representative experiments ($p^* < 0.005$ between two experiments).

adaptive B-cell response, and thereby shortening the time of protection by neutralizing antibodies by 4–5 days [40]. This period of time is critical, if one considers that acute influenza infection can kill mice within a weak. Among the effector CD4 T-cells emerging during a primary immune response to influenza infection, a subset of cells (10–20%) survives and differentiates into long-lasting memory T-cells [41–43]. We next investigated the extent to which the size of $CD4^+Foxp3^+$ T-reg pool affects the primary, memory, and memory-effector T helper cell responses to PR8 virus. Splenocytes from individual mice enriched or not with T-regs 3 days prior to immunization were harvested 14 and 42 days post-immunization and tested for the proliferative capacity and cytokine production upon *in vitro* re-stimulation with $HA_{110-120}$ synthetic peptide (the immunodominant CD4 T-cell epitope of HA of A/PR/8/34 virus) or polyclonal stimulation with Concavalin A (Con A). To reveal significant changes in the adaptive T-cell response to vaccination in relation to small variations in the size of T-reg pool, we chose a higher dose of immunization (200 µg virus protein/mouse) than conventional doses used in BALB/c mice (50–100 µg viral protein/mouse).

Fourteen days post-immunization, the splenocytes from mice immunized with PR8 virus and stimulated *in vitro* with Con A showed a robust proliferative response, whereas those from mice supplemented with T-reg cells prior to immunization showed a reduction in proliferation by 48 to 58% (Fig. 2A). The residual proliferative response of spleen cells detected in the absence of stimulation was most likely due to their *in vivo* activation upon PR8 immunization, since spleen cells from naïve, non-immunized BALB/c mice did not show any residual proliferation in the absence of *in vitro* stimulation (data not shown). The secretion of Th1 and Th2 cytokines was lower in mice enriched for T-regs prior to immunization, although the IL-2 and IL-4 secretion was less affected in the HA-stimulated cells than in cells stimulated with Con A (Fig. 2B). The Th1 suppression was detected as a reduction in IFN-γ secretion upon *in vitro* stimulation. Consistent with the cytokine results, the expression levels of Th1 transcription factors (STAT4 and T-bet) and Th2 transcription factors (STAT6, GATA3, cMAF) in the $CD4^+$ T-cells from T-reg enriched/PR8-immunized mice were lower than in those only immunized with PR8 virus, as determined 14 days post-immunization (Fig. 2C). The primary Th response to PR8 vaccination was also tested in the absence of a pre-existent pool of T-reg cells using BALB/c, RAG2 KO mice reconstituted i.p. with T-reg ($CD25^+$)-depleted splenocytes (74×10^6 cells/mouse) isolated from naïve BALB/c mice, and then immunized 1 day later i.p. with PR8 virus in saline (100 µg viral protein/mouse). The yield of spleen cell reconstitution in RAG mice 9 days after cell infusion was 33–35% ($20–25 \times 10^6$ cells/mouse). Eight days post-immunization, RAG mice reconstituted with T-reg-depleted splenocytes showed a significantly stronger IL-2 and IFN-γ secretion (≈17-times higher) than those reconstituted with whole spleen cells when stimulated *in vitro* for 2 days with PR8 viral proteins (Fig. 2D).

Forty-two days after immunization of naïve BALB/c mice with PR8 virus alone, the splenic CD4 T-cells showed a strong memory-effector Th1 and Th2 cytokine response upon *in vitro* stimulation with Con A (Fig. 3A), and to a lesser extent an $HA_{110-120}$-memory response. The latter response may be due to the fact that the $HA_{110-120}$ epitope accounts for only a small fraction of HA protein antigens. FACS analysis showed also a gradual increase in the size of memory $CD4^+(CD44^{high})$ T-cell pool 14 and 42 days post-immunization in mice immunized with PR8 virus only (Fig. 3B). In contrast, 14 and 42 days post-immunization the splenic CD4 T-cells from T-reg enriched BALB/c mice showed a lower increase in the size of memory $CD4^+44^{high}$ T-cell pool post-immunization than those immunized with PR8 virus alone (27.1% vs. 22.5% 14 days

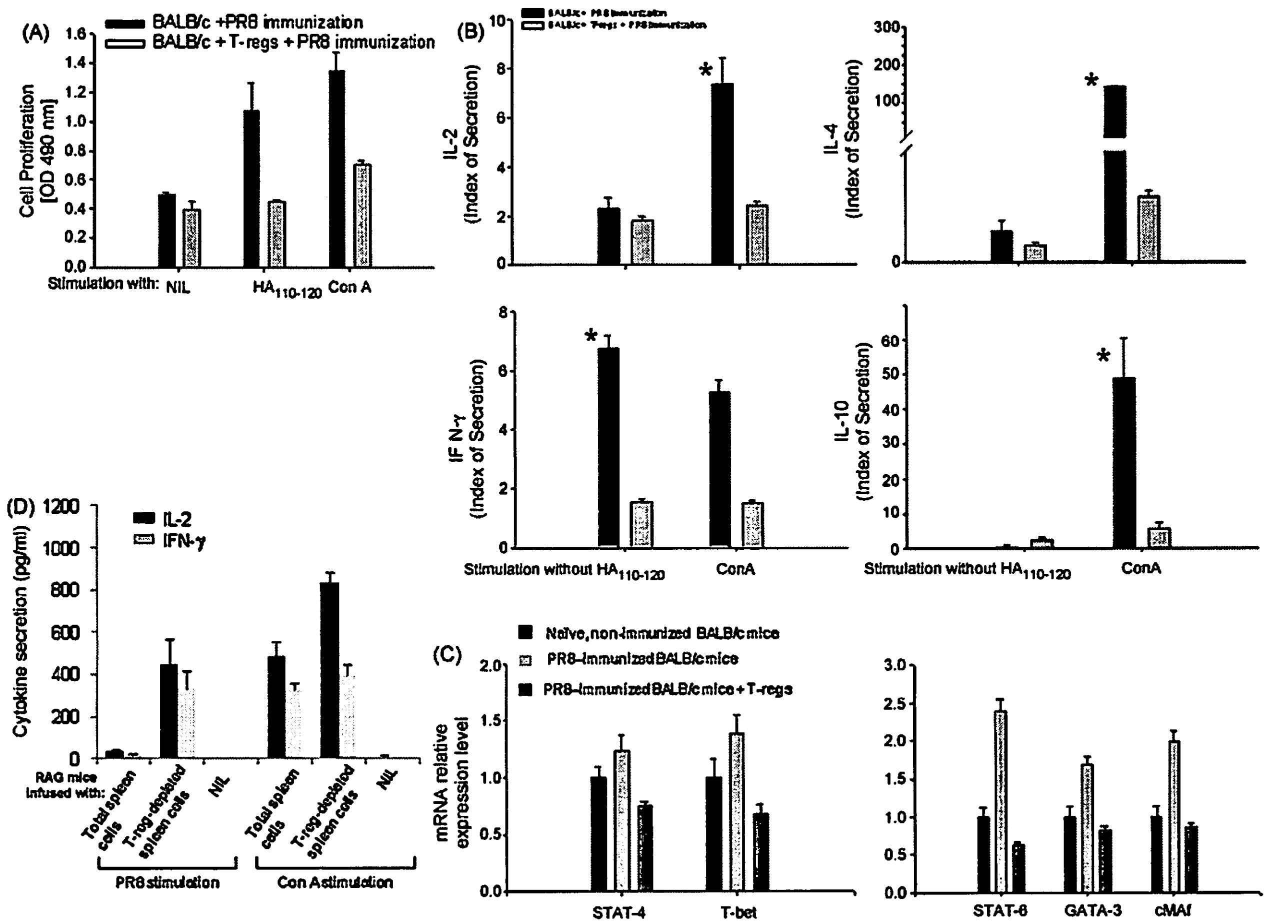


Fig. 2. Effect of T-reg enrichment on the primary T helper cell responses to PR8 viral vaccination. (A) Primary T-cell proliferative response of splenocytes (10^6 cells) from T-reg enriched (6.5×10^6 cells/mouse) or not from BALB/c mice ($n = 6$) immunized with 200 μg of UV-inactivated PR8 viral protein/mouse (14 days post-immunization) upon *in vitro* stimulation with $HA_{110-120}$ synthetic peptide (40 μg/mL) or Con A (2 μg/mL). The OD units refer to the index of proliferation measured at $\lambda = 490$ nm ±SD among triplicate samples from the same experiment. (B) Cytokine production in supernatants from the splenocytes of the same groups of mice upon *in vitro* stimulation with HA peptide or Con A (as in panel A) and measured by Luminex. The index of cytokine secretion is expressed as the pg/mL value of stimulated samples (HA or Con A) divided by the pg/mL value of the non-stimulated samples (NIL, media alone). Results are expressed as the geometric mean of the index of cytokine secretion ±SD among triplicate samples from the same experiment. Shown is (panels A and B) one of two representative experiments ($p^* = 0.002$, between two experiments). (C) Relative mRNA expression of STAT4, T-bet, STAT6, GATA3, and cMAF in aliquot samples from the same groups of mice as in panels A and B and in the absence of *in vitro* re-stimulation. Shown are the mean values ±SD for three individual mice in each group. (D) Cytokine production in cell culture supernatants of splenocytes from BALB/c, RAG2 KO mice pre-infused with 74×10^6 cells/mouse of either total spleen cells ($n = 3$), or T-reg-depleted spleen cells ($n = 3$), or saline ($n = 3$), and then immunized 1 day later i.p. with inactivated PR8 virus (100 μg/mouse). Spleen cells of RAG2 KO recipients were collected 8 days post-immunization, stimulated for 2 days *in vitro* with purified PR8 virus (10 μg viral protein/10^6 cells) or ConA (1 μg/10^6 cells), and cytokine secretion in culture supernatants was measured by Luminex. The signal-to-noise from aliquot wells left non-stimulated was subtracted from each sample. Results from individual mice are expressed as mean pg/mL ±SD among triplicate samples from the same experiment. Shown are the results of one of two representative experiments ($p^* = 0.004$ between experiments).

post-immunization, and 33.6% vs. 24% 42 days post-immunization) (Fig. 3B), and a reduction in the memory-effector Th1 and Th2 function upon *in vitro* stimulation as detected in the cytokine assays.

Furthermore, to measure the extent to which $CD4^+$ memory-effector T-cell function is affected *in vivo* by $Foxp3^+$ T-reg cells induced through PR8 vaccination, we took advantage of a murine reporter system in which the PR8/HA-specific T-effector cells induce autoimmune diabetes. In this system, adoptively transferred $HA_{110-120}$-specific CD4 T-cells (TCR-PR8/HA) into RAG2 KO mice expressing the HA of PR8/A/34 influenza virus in the pancreatic β-cells (RAG2 KO, RIP-PR8/HA Tg mice) lead to fulminate autoimmune diabetes within two weeks, as depicted by hyperglycemia and heavy infiltration of pancreatic β-islets with lymphocytes (pancreatic insulitis) (Fig. 4A) [44]. These mice can survive up to 1 month after the hyperglycemia onset. Similarly, the RAG2 KO, RIP-PR8/HA Tg mice infused with $CD4^+$ memory T-cells from PR8-immunized BALB/c mice harvested 42 days post-immunization developed hyperglycemia, although with a delay of 2–15 days, and longer survival (till 2 months) after hyperglycemia onset (Fig. 4B). Development of diabetes was indicative for activation of PR8-induced, HA-specific T memory cells toward the effector function upon encountering the PR8 viral antigens in the pancreas of RAG2 KO, RIP-PR8/HA Tg recipient mice. A short delay in diabetes onset in this group of mice may well account for the lag period required by HA memory T-cells to develop into effector cells, to which the PR8-induced T-reg cells may have contributed by suppression of HA-specific T memory-effector cells. The recipient mice in this group also showed pancreatic insulitis, as indicated by the hematoxylin-eosin (HE) staining of pancreatic sections 50 days after the cell transfer (Fig. 4B).

In contrast to this group of mice, the RAG2 KO, RIP-PR8/HA Tg mice infused with memory CD4 T-cells from BALB/c mice enriched in T-regs prior to PR8 immunization remained normoglycemic for over 70 days (Fig. 4C). Normoglycemia was maintained even after co-infusion of TCR-PR8/HA diabetogenic T-cells (2×10^5 cells/mouse). Histological analysis of the pancreas of protected (normoglycemic) mice harvested 50 and 70 days after cell trans-

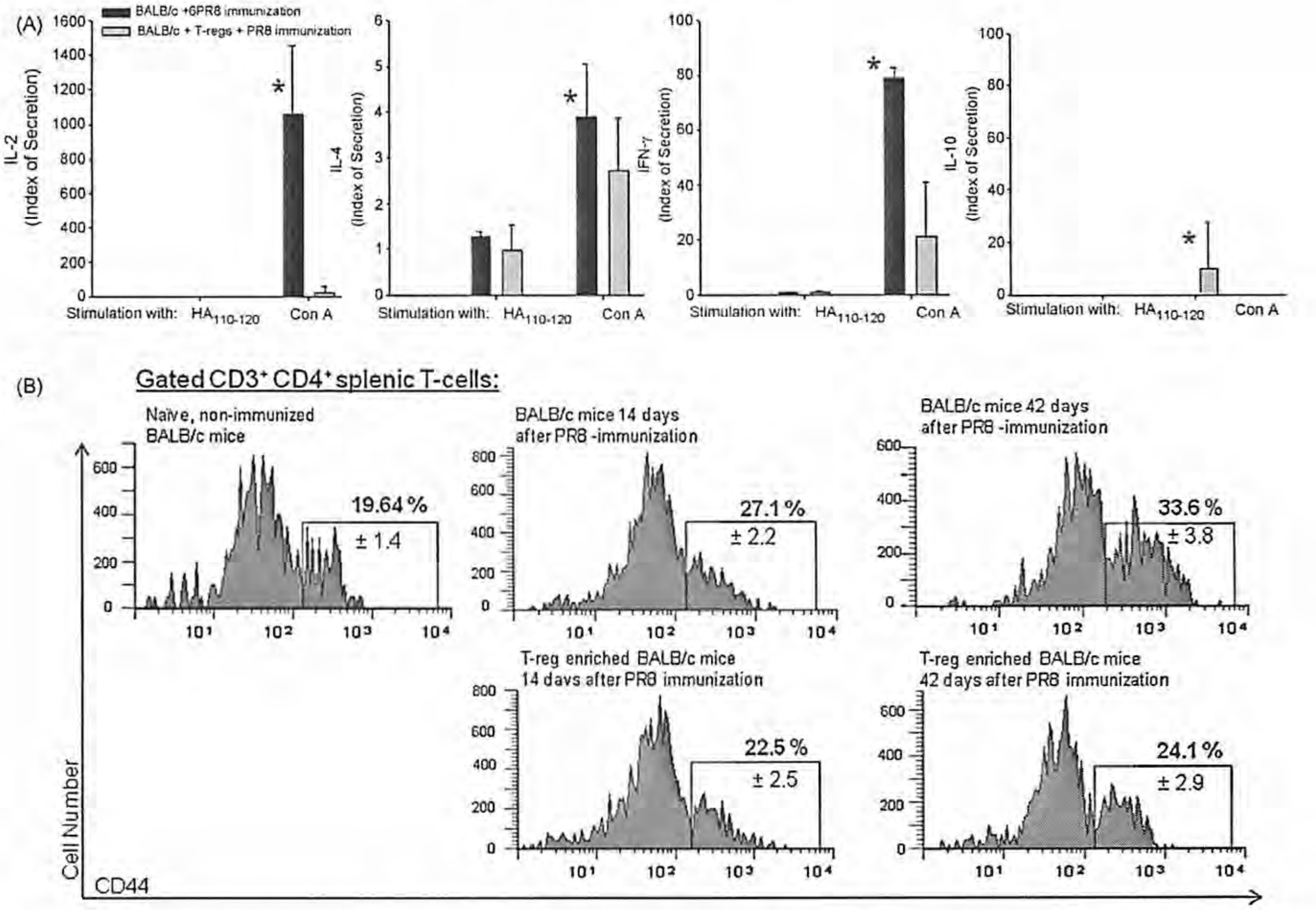


Fig. 3. Effect of T-reg enrichment on the memory T helper cell response to PR8 viral vaccination. (A) Cytokine production in supernatants from the splenocytes (10^6 cells) of T-reg enriched (6.5×10^6 cells/mouse) or not from BALB/c mice ($n = 6$) immunized with 200 μg of UV-inactivated PR8 virus/mouse (42 days post-immunization) upon *in vitro* re-stimulation with $HA_{110-120}$ synthetic peptide (40 μg/mL) or Con A (2 μg/mL) and measured by Luminex. The index of cytokine secretion is expressed as the pg/mL value of stimulated samples ($HA_{110-120}$ peptide or Con A) divided by the pg/mL value of the non-stimulated samples (NIL, media alone). Results are expressed as the geometric mean of the index of cytokine secretion ±SD among triplicate samples from the same experiment. Shown is one of two representative experiments ($p^* = 0.0042$ between two experiments). (B) Kinetics in the frequency of $CD4^+CD44^{high}$ memory T-cells from the splenic cells of T-reg enriched (6.5×10^6 cells/mouse) or not from BALB/c mice ($n = 6$) immunized or not with 200 μg of UV-inactivated PR8 viral protein/mouse, before, and after 14 and 42 days post-immunization. Cells were stained with CD3-FITC, CD4-PerCP, and CD44-PE Ab conjugates (BD PharMingen) and the number of $CD3^+CD4^+CD44^{high}$-gated cells was measured by FACS among 10^5 cell events. Shown are individual mice ($n = 6$) from each group of mice ($p < 0.005$ between individual mice).

fer showed a low degree of pancreatic insulitis. When tested for the insulin secretory function of pancreatic β-cells by the glucose tolerance test, the protected mice showed a sharp decline (within an hour) from a hyperglycemic-induced status to a normoglycemic status, just as the naïve RAG2 KO, RIP-PR8/HA Tg mice did (Fig. 5A and D). In contrast, the RAG2 KO, RIP-PR8/HA Tg recipients of CD4 memory T-cells from PR8-only immunized BALB/c mice tested by the glucose tolerance test remained in a hyperglycemic state for 3 h (Fig. 5C) as compared with those infused only with TCR-PR8/HA diabetogenic T-cells (diabetes control group) in which hyperglycemia persisted longer than 3 h after the glucose load (Fig. 5B). These data indicated first that PR8 vaccination induced HA-specific memory CD4 T-cells able to develop into HA-specific T-effector cells upon encountering the PR8/HA protein antigens in the pancreas of RAG2 KO, RIP-PR8/HA Tg mice. Second, the T-reg cells induced by PR8 immunization were fully functional, as they efficiently suppressed the PR8/HA-specific (diabetogenic) T-cells in this system. The suppressive effect of PR8-induced T-regs on TCR-PR8/HA diabetogenic T-cell function was stronger when the size of T-reg pool was enriched in BALB/c donors prior to vaccination.

These results strongly suggest that the size of $CD4^+Foxp3^+$ T-reg pool is a critical modulatory component of the adaptive (PR8 virus-specific) $CD4^+$ T-cell primary and memory responses induced by vaccination.

3.3. Influenza vaccination favors expansion of $CD4^+Foxp3^+$ T-reg cells

We next questioned whether the PR8 vaccination alters the number of T-reg cells. For this, we compared T-reg frequency during the primary and memory T helper immune responses to PR8 virus after a single high-dose immunization (200 μg viral protein/mouse) with that of T-regs in BALB/c mice enriched or not with naive BALB/c $CD4^+Foxp3^+$ T-regs prior to immunization. Data from naïve, non-immunized BALB/c mice supplemented or not with $Foxp3^+$ T-regs in the absence of immunization showed a relative constant frequency of $Foxp3^+$ cells among the $CD4^+$ T-cell pool (2.8–3.2% and 3.8–4.4% $CD4^+Foxp3^+$ cells, respectively) during a 42-day follow-up (Fig. 6). This indicated that in the absence of immunization the homeostatic expansion of peripheral $Foxp3^+$ T-reg cells was insignificant. However, 14 days post-immunization with PR8 virus alone, the number of $CD4^+Foxp3^+$ cells increased by ~28% and continued to increase 42 days later by ~100% (Fig. 6A), which clearly indicated an expansion of $Foxp3^+$ T-reg pool induced by PR8 immunization. The BALB/c

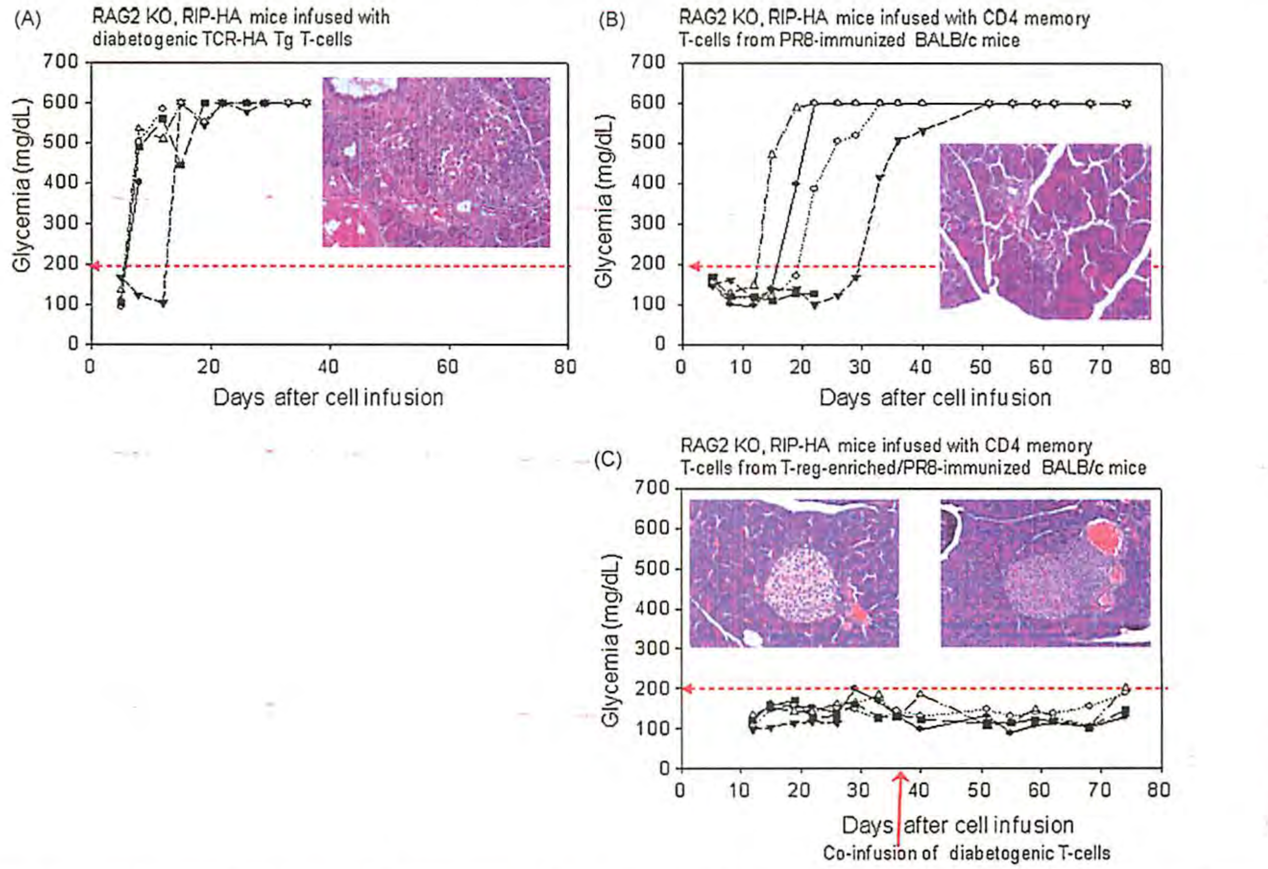


Fig. 4. The suppressogenic function of T-reg cells upon PR8 viral vaccination. Groups of RAG2 KO, RIP-PR8/HA Tg mice ($n = 5$ mice/group) were infused i.p. with splenic TCR-PR8/HA CD4 diabetogenic T-cells (2×10^5 cells/mouse, diabetes control group) (panel A), or with splenic CD4$^+$ memory T-cells from PR8 immunized BALB/c mice (2×10^5 cells/mouse) harvested 42 days post-immunization) (panel B). Inserts show the H&E staining of a representative pancreatic section from one recipient mouse in each group, 30 and, respectively, 60 days after cell transfer. Glycemia values are indicated for individual mice in each group. Upper limit of normoglycemia (200 mg/dL, dotted line) was previously determined on a large cohort of naïve RAG2 KO, RIP-PR8/HA Tg mice after overnight fastening. (C) RAG2 KO, RIP-PR8/HA Tg mice ($n = 5$) were infused i.p. with splenic CD4$^+$ memory T-cells from T-reg enriched/PR8-immunized BALB/c mice harvested 42 days post-immunization. Thirty-five days after cell infusion (dark arrow) mice were co-infused i.p. with TCR-PR8/HA diabetogenic T-cells (2×10^5 cells/mouse) and glycemia monitored on a bi-weekly basis. Shown are the H&E staining of pancreatic sections from one representative naïve (non-infused) RAG2 KO, RIP-PR8/HA Tg mouse that lacks pancreatic infiltration (left insert), and one representative RAG2 KO, RIP-PR8/HA Tg mouse 35 days after co-infusion of TCR-PR8/HA diabetogenic cells showing a limited, peri-islet infiltration with lymphocytes (right insert).

mice enriched with T-regs prior to immunization showed a higher increase in the number of CD4$^+$Foxp3$^+$ T-reg cells 14 and 42 days post-immunization (~39% to ~ 73%) (Fig. 6B). These data clearly indicated that the size of CD4$^+$Foxp3$^+$ T-reg pool was expanded after PR8 immunization proportionally to its size prior to immunization.

Alternatively, we used a subtractionist approach to measure the *in vivo* T-reg kinetics upon PR8 viral vaccination. In this system, BALB.c/RAG2 KO mice were first infused with naïve T-reg (CD25$^+$)-depleted spleen cells or total spleen cells (74×10^6 cells/mouse) from BALB/c mice, then immunized with PR8 virus 1 day later, and Foxp3 mRNA expression was compared 8 days post-immunization in the spleen cells by real-time RT-PCR (Fig. 6C). While the non-reconstituted RAG mice that were immunized with PR8 virus showed a lack of Foxp3 mRNA expression, those reconstituted with T-reg-depleted spleen cells and immunized with PR8 virus showed the presence of a small amount of Foxp3 mRNA. Also, the pre-existent Foxp3 mRNA expression in RAG mice pre-infused with total spleen cells was increased by ≈18% upon PR8 vaccination.

Together, these results indicated that PR8 vaccination expands the pool of T-reg cells, and at the same time, a fraction of T-reg cells differentiate from the naïve conventional T-cell pool following PR8 vaccination.

3.4. *Influenza vaccination favors the development of PR8 (HA)-specific Foxp3$^+$ T-reg cells*

Recent data from individuals vaccinated for flu showed that CD4$^+$CD25$^-$ T-cells can differentiate into fully functional, antigen (HA)-specific CD4$^+$CD25$^+$(Foxp3$^+$) T-reg cells upon stimulation *in vitro* with influenza $HA_{306-318}$ peptide [45]. It is believed that the antigen-specific T-reg suppression of cognate T-effector cells is more powerful than a non-specific bystander suppression induced by T-regs with unrelated antigen-specificity or naïve T-reg cells that have not experienced the antigen. To test the antigen specificity of T-reg cells induced *in vivo* by PR8 vaccination, we immunized groups of naïve or T-reg enriched BALB/c mice with the same dose of UV-inactivated BHK influenza strain, and compared the capacity of BHK- vs. PR8-induced T-reg cells to suppress PR8/HA-specific T-cells that induce diabetes in the BALB/c, RAG2 KO, RIP-PR8/HA transgenic mouse. The BHK virus lacks the PR8/HA antigens. Immunization of mice with BHK virus induced similar titers of HAI Abs (1/320–1/640) to those detected in PR8-immunized mice (1/160–1/320). The RAG2 KO, RIP-PR8/HA Tg mice infused only with CD4$^+$ T-cells alone (2×10^5 cells/mouse) from BHK-immunized BALB/c mice 14 days post-immunization remained free of diabetes (data not shown), indicating that the HA-specificity of T-effector cells induced by BHK immunization is

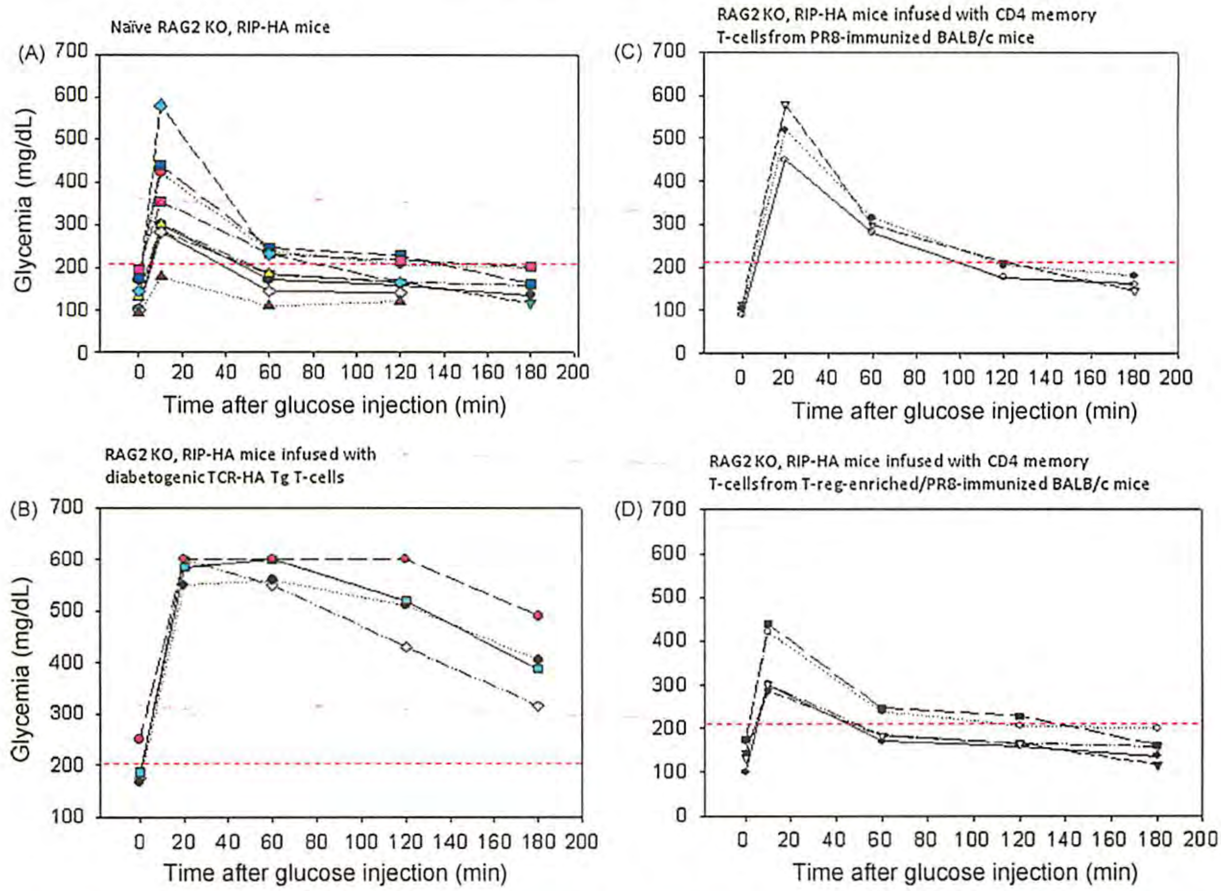


Fig. 5. Glucose tolerance test in RAG2 KO, RIP-PR8/HA Tg recipient mice of BALB/c PR8-specific T-regs induced by vaccination. (A) Naïve RAG2 KO, RIP-PR8/HA Tg mice ($n = 7$, normoglycemia control group), or infused i.p. with splenic TCR-PR8/HA diabetogenic T-cells ($n = 4$, diabetes control group) (panel B), or RAG2 KO, RIP-PR8/HA Tg mice ($n = 3$) infused i.p. with splenic CD4$^+$ memory T-cells (2×10^5 cells/mouse) from BALB/c mice immunized with PR8 virus and harvested 42 days post-immunization (panel C), or RAG2 KO, RIP-PR8/HA Tg mice ($n = 3$) infused i.p. with splenic CD4$^+$ memory T-cells (2×10^5 cells/mouse) from T-reg enriched, BALB/c mice immunized with PR8 virus and harvested 42 days post-immunization (panel D) were fasted overnight and then injected i.p. with glucose in saline (60 mg/mouse). Blood glucose levels were determined at various intervals of time during a 180-min period after glucose load. Upper limit of normoglycemia (200 mg/dL) in naïve RAG2 KO, RIP-PR8/HA Tg mice after overnight fastening is indicated (dotted line).

unrelated to the HA-specificity of T-effector cells induced by PR8 immunization. Also, co-infusion of 2×10^5 TCR-PR8/HA-specific (diabetogenic) T-cells together with memory CD4$^+$ T splenic cells (2×10^5 cells/mouse) harvested 42 days post-BHK immunization of mice enriched or not with the same number (6.5×10^6 cells/mouse) of naïve BALB/c T-reg cells, did not protect against diabetes. These mice developed diabetes within the same period of time (2 weeks) and showed lymphocyte infiltration of pancreatic β-islets as those infused only with 2×10^5 TCR-PR8/HA-specific (diabetogenic) T-cells (diabetes control group) (Fig. 7). These results showed that the BHK HA-specificity of T memory-effector cells as well as T-reg cells was unrelated to the PR8/HA-specificity. Only the PR8-induced T-reg cells were able to protect against diabetes in this mouse model for PR8/HA-induced diabetes (Fig. 4), which demonstrated that the suppressogenic effect of T-reg cells induced by PR8 vaccination was restricted to the T-effector cells specific for HA protein of PR8/A/34 influenza strain. These experiments demonstrated that a fraction of T-reg cells induced by PR8 vaccination were specific for PR8/HA antigens.

4. Discussion

Human HA-specific CD4$^+$CD25$^+$Foxp3$^+$ T-regs can be generated *in vitro* from naïve and memory CD4$^+$CD25$^-$ T-cells of individuals previously vaccinated for flu by a 10-day re-stimulation *in vitro* with dendritic cells pulsed with influenza $HA_{306-319}$ consensus peptide [45]. T-reg suppression of antigen (HA)-specific T-effector cells as well as non-specific bystander suppression of T-effector cells specific for tetanus toxoid antigens was only achieved in the presence of $HA_{306-319}$ cognate peptide, implying that Foxp3$^+$ T-reg cells require antigen stimulation to gain both antigen-specific and non-specific bystander suppressive functions [45]. The CD4$^+$Foxp3$^+$ T-regs are naturally born in thymus by differentiation of T-cell precursors upon TCR stimulation by MHC-peptide complexes [46]. Like the human T-reg cells [45], the murine naïve CD4$^+$Foxp3$^-$ T-cells can up-regulate Foxp3 expression and gain suppressogenic function in periphery upon encountering antigen in the presence of TGF-β [46], or IL-2 and IL-15 [47,48].

A body of evidence showed that T-regs can efficiently suppress autoimmunity including type 1 diabetes. Using a BALB/c, RAG2 KO, RIP-PR8/HA transgenic mouse model in which infusion of influenza PR8/HA-specific T-effector cells (from a TCR-PR8/HA Tg mouse) induces fulminate diabetes, we found that the CD4$^+$CD25$^+$(Foxp3$^+$) T-regs induced in BALB/c mice through vaccination with influenza PR8 virus, abolished the diabetogenic function of PR8/HA-specific T-effector cells for as long as 70 days. A fraction of Foxp3$^+$ T-regs induced by PR8 vaccination were HA-specific, since the T-regs induced by vaccination with BHK virus expressing a structurally different HA protein did not abolish the diabetogenic function of PR8/HA-specific T-effector cells in the RAG2 KO, RIP-PR8/HA mouse system. This clearly indicated that the PR8/HA-specific Foxp3$^+$ T-

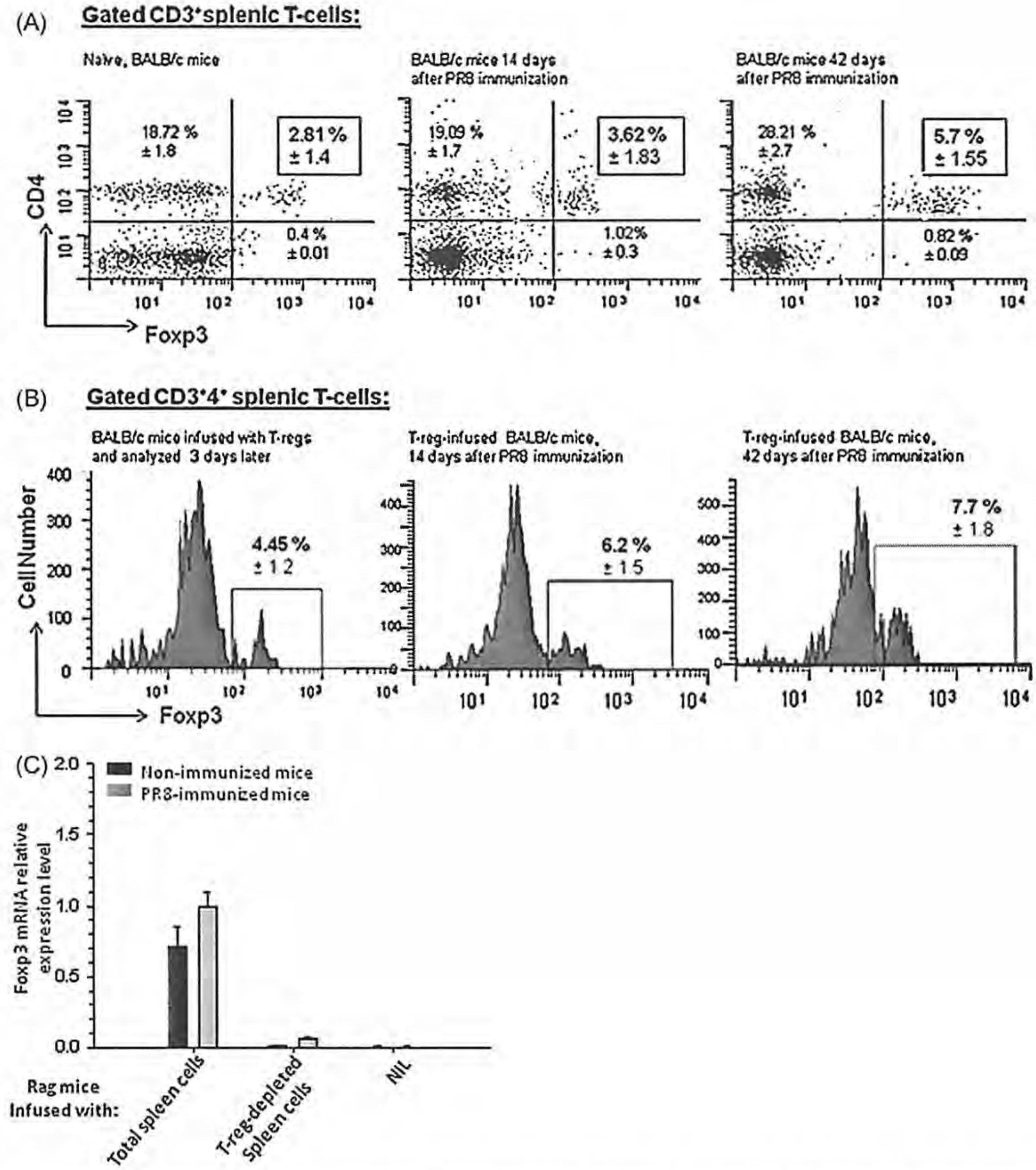


Fig. 6. Frequency of CD4+Foxp3+ T-regs upon T-reg enrichment in naïve BALB/c mice before and after PR8 viral vaccination. Splenic T-cells from BALB/c T-reg enriched (panel B) or not (panel A) that were immunized or not with 200 μg of UV-inactivated PR8 viral protein/mouse were surface stained with CD3-FITC, CD4-PerCP and intra-nuclear stained with Foxp3 Ab-PE conjugate 14 and 42 days post-immunization. Shown are the mean values for individual mice (framed histograms) in each group of mice indicating the percent of Foxp3+ T-reg cells at various time-points ±SD ($n = 6$ mice/group) in one of two representative experiments ($p^* < 0.005$ between experiments). (C) Foxp3 mRNA expression in splenocytes collected from BALB/c, RAG2 KO mice pre-infused with 74×10^6 cells/mouse of either total spleen cells ($n = 3$), or T-reg-depleted spleen cells ($n = 3$), or saline ($n = 3$), and then immunized with PR8 virus in saline 1 day later (100 μg/mouse). Spleen cells of RAG2 KO recipients were collected 8 days post-immunization, and Foxp3 mRNA expression was determined 8 days post-immunization in individual mice by real-time RT-PCR. Results are expressed as mean of relative mRNA expression from individual mice ±SD in one of two representative experiments ($p^* = 0.005$ between experiments).

regs suppressed more efficiently their cognate T-effector cells *in vivo* than the T-reg bystander suppressors specific for BHK/HA antigens.

Vaccination of BALB/c mice with a single high-dose of a prototype UV-inactivated influenza A/PR/8/34 virus vaccine expanded the Foxp3+ T-reg pool by almost 30 and 100%, 14 and, respectively, 42 days post-vaccination. A two-fold increase in the size of Foxp3+ T-reg pool over the pre-existent pool size (before vaccination) did not alter the PR8-specific B-cell responses, but it did lower significantly the primary and memory PR8-specific T helper responses to the virus. Conversely, mice depleted of T-regs mounted a significantly higher T helper response to PR8 viral vaccination than those with a pre-existent T-reg pool. Interesting enough, a small fraction of newly generated T-reg cells from naïve conventional T-cells was detected post-vaccination in mice depleted of T-regs, which is consistent with a number of reports indicating that T-regs may differentiate from peripheral naïve CD4+Foxp3+T-cells upon encountering antigen in a milieu of TGF–β, or IL-2 and IL-15 [46–48].

B memory cell responses specific for influenza HA viral proteins are particularly long-lived, as demonstrated by studies in elderly individuals after several decades from the virus expo-

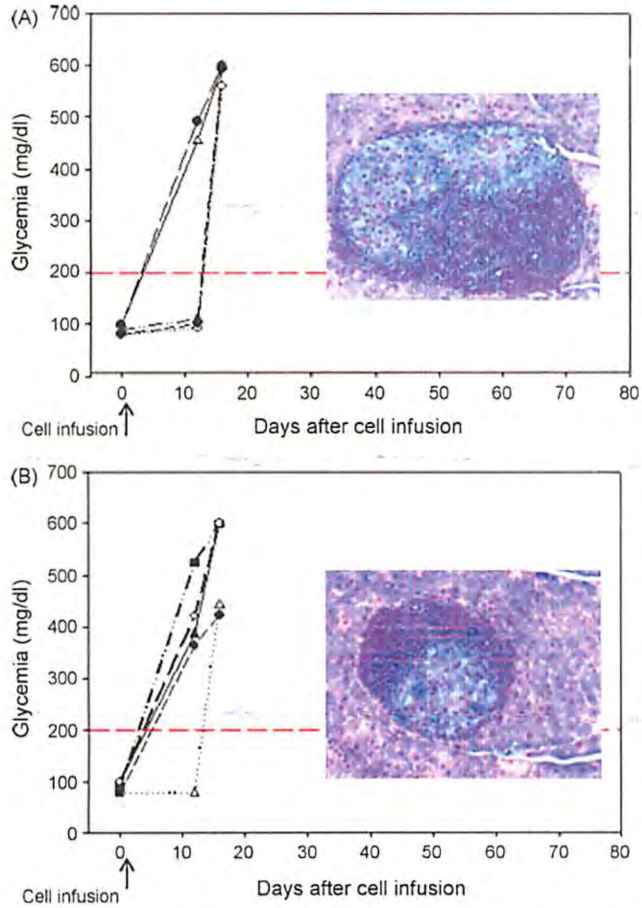


Fig. 7. Antigen-specificity of CD4⁺Foxp3⁺ T-regs induced by PR8 viral vaccination. Panel A: RAG2 KO, RIP-PR8/HA Tg mice ($n = 3$) co-infused i.p. with TCR-PR8/HA diabetogenic cells (2×10^5 cells/mouse) and CD4⁺ splenic T-cells (2×10^5 cells/mouse) from BHK-immunized BALB/c mice. Panel B: RAG2 KO, RIP-PR8/HA Tg mice ($n = 5$) co-infused i.p. with TCR-PR8/HA diabetogenic cells (2×10^5 cells/mouse) and CD4⁺ splenic T-cells (2×10^5 cells/mouse) from BHK-immunized BALB/c mice and enriched in T-reg cells (6.5×10^6 cells added/mouse) prior to immunization. The CD4 splenic T-cells from BHK-immunized BALB/c mice were collected 42 days after immunization. Plots indicate the level of blood glucose in individual mice till reaching the hyperglycemic status. Inserts in panels A and B show the H&E staining of pancreatic sections from one representative recipient mouse in each group. As illustrated, both groups of mice showed heavy pancreatic infiltration of β-islets with lymphocytes 35 days after cell co-infusion. Dotted line indicates the upper limit of normoglycemia (200 mg/dL).

sure [49]. The HA-specific B memory cells are critical for clearing the virus from the lungs, as they are activated to secrete neutralizing antibodies subsequent to repeated infections with the same virus or cross reactive viruses [50]. Upon encountering viral antigens, the CD4 T-cells are activated and differentiate into antigen (virus)-specific Th cells. A large number of these cells die by activation-induced apoptosis, whereas a smaller number enter the peripheral lymphoid organs to become memory Th cells [51]. Upon the antigen recall following a viral infection or vaccination boost, the virus-specific memory T helper cells rapidly become memory-effector cells [52,53]. Influenza-specific T memory cells induced by vaccination or exposure to the live virus do not clear efficiently the virus in the absence of antibodies [54], but they do contribute significantly to the anti-viral protective immunity by shortening the time of recall for B memory cell activation leading to secretion of neutralizing antibodies. A delay of 4 to 5 days in virus clearance from the lungs was observed in CD4-depleted mice, which was essential for recovery from infection and survival (reviewed in ref [55]). We noticed that PR8 vaccination led to increased number of CD4⁺CD44^{high} T memory cells 14 and 42 days post-immunization following similar kinetics to those reported for memory T-cells activated upon re-infections [56,57]. In contrast to influenza-specific B-cell responses, the virus-specific Th1 and Th2 primary and memory responses were significantly suppressed by the PR8 vaccination-induced CD4⁺Foxp3⁺T-reg cells.

In summary, our findings showed that the size of CD4⁺Foxp3⁺ T-reg pool is an important modulatory component of primary and memory T-cell responses to influenza vaccination. Since the CD4 T-cells play an important role in anti-influenza viral protection through a quick recall of the adaptive B memory cell response, and T-regs are expanded as consequence of vaccination, one may explain at least in part why not all vaccinated individuals are resistant to influenza exposure. Individual variations in the size of T-reg compartment in various ethnic groups were so far correlated with susceptibility to autoimmune diseases [58]. Thus, attempts to deplete T-reg cells prior to vaccination [59] or to abolish T-reg suppressive function by GITR blocking Ab [60,61] or by manipulating expression of sphingosine 1-phospatate receptor type 1 ($S1P_1$) [62] may raise safety concerns due to the increased risk of autoimmunity [63]. Early studies on influenza virus suggested that hypothetical suppressogenic epitopes expressed among immunogenic epitopes of the viral envelope are responsible for induction of antigen-specific T "suppressor" cells, and that down-modulation of such suppressogenic epitopes might help designing new vaccine preparations able to increase the resistance to infection [11]. However, it has been later demonstrated in various antigen-specific systems that the immunogenic epitopes are structurally identical to the suppressogenic ones [7] and thereby, the concept of "epitope structural identity" may greatly impact the vaccinology field by generating new influenza viruses with random mutations in HA protein. Such newly generated (mutated) viral epitopes may outsmart antigen-drifting that occurs on a regular basis among the influenza strains, although they may lack not only suppressogenic epitopes but also immunogenic epitopes required for induction of protective immunity. Our basic knowledge about "regulation" of T-reg cells is in infancy, but new findings suggest that the dendritic cells may be used to turn-off T-reg function through mechanisms engaging IL-6, TLRs 4, or TLR9 [64,65]. Without a doubt, investigations on the therapeutic "quenching" of T-reg function prior to vaccination is an exciting area that may lead to the development of anti-viral vaccines endowed with better efficacy.

Acknowledgements

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